VIROLOGY 212, 340–347 (1995)

Suppression of Influenza Virus Infection by an *N*-Thioacetylneuraminic Acid Acrylamide Copolymer Resistant to Neuraminidase

MASAE ITOH,* PETER HETTERICH,† RAINER ISECKE,† REINHARD BROSSMER,† and HANS-DIETER KLENK*,[1]

*Institut für Virologie, Klinikum der Philipps-Universität Marburg, Robert-Koch Strasse 17, 35037 Marburg, Germany; and †Institut für Biochemie II, Ruprecht-Karls-Universität Heidelberg, Im Neuenheimer Feld 328, 69120 Heidelberg, Germany

Received March 1, 1995; accepted June 28, 1995

We have previously shown that α-2-*O*-methyl-5-*N*-thioacetylneuraminic acid (α-Neu5thioAc2Me) has a higher affinity to bromelain-treated hemagglutinin (HA) of influenza A virus than sialic acid from natural sources (Machytka *et al.*, 1993, *FEBS Lett.* 334, 117–120). We have now compared the inhibitory effects of α-Neu5thioAc2Me and other sialic acid analogs on receptor binding and plaque formation of intact influenza A viruses. When α-Neu5thioAc2Me was polymerized by conjugation to polyacrylamide, its affinity to HA increased 10^3-fold. When analyzed by plaque reduction, the α-Neu5thioAc2 polymer was about 10 times more efficient as an inhibitor of virus replication than the α-Neu5Ac2 polymer, stressing the importance of sulfur at C5. The *S*-glycoside α-2-*S*-methyl-5-*N*-thioacetylneuraminic acid (α-Neu5thioAc2SMe) had the same affinity to HA as α-Neu5thioAc2Me, but was resistant to neuraminidase. The α-Neu5thioAc2S polymer interfered with the replication of a wider spectrum of influenza A virus subtypes than the α-Neu5thioAc2 polymer. The results indicate that the α-Neu5thioAc2S polymer has the potential to be used as an inhibitor of influenza virus infection. © 1995 Academic Press, Inc.

INTRODUCTION

Influenza viruses express two envelope proteins, hemagglutinin (HA) and neuraminidase (NA). HA mediates cell recognition by binding to cell surface receptors that are the terminal sialic acid moieties of glycoproteins or gangliosides (Paulson *et al.*, 1979; Bergelson *et al.*, 1982; Paulson, 1985; Suzuki *et al.*, 1985). NA is presumably responsible for the elution of progeny virus by removal of sialic acid from the surface of infected cells.

The molecular basis of the interaction between the receptor binding site (RBS) situated at the distal end of HA and sialic acid has been intensively studied in several laboratories. Thus, the three-dimensional structure of two H3 HAs (X-31 and X-31/HS) complexed with the natural receptor analog sialyllactose has been resolved by X-ray analysis, and possible atomic interactions of Neu5Ac with HA have been predicted from the corresponding interatomic distances (Weis *et al.*, 1988). The affinities of a number of sialic acid analogs for HA were also investigated by binding experiments to evaluate which parts of the Neu5Ac are essential for binding, and the importance of the carboxylic, *N*-acetyl, C7-, and C9-hydroxyl groups of Neu5Ac in ligand binding was confirmed (Pritchett *et al.*, 1987; Sauter *et al.*, 1989, 1992; Kelm *et al.*, 1992). It was also demonstrated that viruses are distinctive in the recognition of oligosaccharide sequences with the different types of sialic acid linkages to the penultimate galactose (Neu5Acα2-3Gal or Neu5Acα2-6Gal) and with the different core structures bearing the same Neu5Ac-Gal linkage (Rogers and Paulson, 1983; Pritchett *et al.*, 1987; Sauter *et al.*, 1989, 1992; Nobusawa *et al.*, 1991; Suzuki *et al.*, 1992; Matrosovich *et al.*, 1993). The data clearly indicate that the ability of sialic acid to serve as a receptor determinant of influenza virus is influenced by the carbohydrate structure to which it is attached.

Prevention of influenza virus infection by vaccination is hampered by the antigenic drift and shift of HA and NA (Laver and Kilbourne, 1966). One of the most promising alternatives to interfere with virus infection is blocking of receptor binding. Three possible ways have been proposed for designing high-affinity inhibitors. The first approach is modification of the functional groups of the Neu5Ac moiety to increase its intrinsic affinity (Pritchett *et al.*, 1987; Sauter *et al.*, 1992). The second approach is to construct multisialylated polymers. Monomeric sialosides such as α-2-*O*-methyl-5-*N*-acetylneuraminic acid (Neu5Ac2Me) or α-(2-3)-sialyllactose bind to HA incorporated into virus particles or to solubilized HA with the relatively high dissociation constant of 2 m*M*. However, polyvalent sialic acid obtained by conjugation to proteins or synthetic polymers has enhanced binding affinity against virus (Morawiecki and Lisowska, 1965; Whitehead and Winzler, 1968; Barclay *et al.*, 1969; Hanaoka *et al.*, 1989; Pritchett and Paulson, 1989; Matrosovich *et al.*, 1990; Glick and Knowles, 1991; Sabesan *et al.*, 1991; Spaltenstein and Whitesides, 1991). A single influenza virus particle contains about 500 HA molecules

[1] To whom reprint requests should be addressed. Fax: 06421/28-8962.

0042-6822/95 $12.00

(Tiffany and Blough, 1970; Ruigrok *et al.*, 1984), and the increased affinity of polyvalent sialosides could be explained by simultaneous multipoint binding events between sialosides and HA on virus. The third way is to make sialosides resistant against neuraminidase. It is well known that equine and guinea pig α2-macroglobulin, which contain 4-*O*-acetyl-5-*N*-acetylneuraminic acid (Neu4,5Ac_2), inhibit the receptor binding of influenza virus more effectively than human α2-macrogloblin, which does not possess this type of sialic acid. Although the binding affinities of Neu5Ac and Neu4,5Ac_2 for HA are almost equal, Neu4,5Ac_2 is partially resistant to virus NA (Pritchett and Paulson, 1989). Resistance to NA may therefore be an important property to be considered with inhibitor design.

On the basis of the available information on the molecular details of the interaction of Neu5Ac with HA, we recently developed a number of Neu5Ac analogs which included α-2-*O*-methyl-5-*N*-thioacetylneuraminic acid (Neu5thioAc2Me). As indicated by ^{1}H NMR analysis, the compound exhibited a dissociation constant (K_d) of 0.1 m*M* against bromelain-treated HA. It is, thus, the first simple sialoside monomer that has significantly higher affinity toward influenza HA than the natural receptors (Machytka *et al.*, 1993). In the present study, we describe the interference of Neu5thioAc2Me with receptor binding of virus particles. Furthermore, we have analyzed the effects of polymers of Neu5thioAc with virus replication and of derivatives designed to make them resistant against NA degradation.

MATERIALS AND METHODS

Viruses

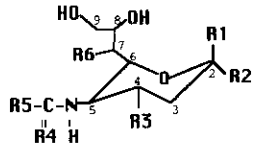
Influenza viruses were grown in the allantoic cavity of 10-day-old embryonated eggs. Allantoic fluid was harvested and stored at −80°. For competitive binding experiments and viral NA treatment of sialic acid analogs, the A/Aichi/2/68 (X-31) strain of influenza A virus was purified by 5−50% sucrose gradient centrifugation.

Sialic acid analogs, monomers, and polymers

The structures of sialic acid analogs used in this study are shown in Fig. 1. If not indicated otherwise, abbreviations for sialic acid analogs refer to the α-anomeric compounds. α-Neu5Acryl2Me and α-Neu5Mecar2Me were prepared from α-2-*O*-methyl neuraminic acid (Isecke and Brossmer, 1994) using acryloyl chloride and methyl isocyanate, respectively. Details will be given elsewhere (P. Hetterich and R. Brossmer, manuscript in preparation). α-Neu5Prop2Bn and α-Neu5FAc2Bn were prepared from α-2-*O*-benzyl neuraminic acid using propionic anhydride and fluoroacetic anhydride, respectively. α-Neu4,5-$Ac_2$2Bn and α-Neu4,5,7$Ac_3$2Bn were synthesized from α-2-*O*-benzyl-5*N*-acetyl-8,9-*O*-isopropylidene−neuraminic acid. α-Neu5Ac2Me and β-Neu5Ac2Me (Kuhn *et al.*, 1966) as well as α-Neu5Prop2Me, α-Neu5thioAc2Me, α-Neu5thioProp2Me, and α-Neu5thioAc2SMe (Isecke and Brossmer, 1994) were synthesized as described.

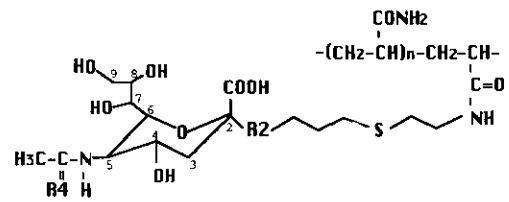
For preparation of the sialic acid analog-containing polymers the α-*O*- or α-*S*-allyl glycoside of 5-*N*-thioacetylneuraminic acid or the α-*O*-allyl glycoside of 5-*N*-acetylneuraminic acid was synthesized. Addition of aminoethanethiol to the allylic double bond and subsequent acroylation afforded the final aglycon, the 3-(2-acryl-

A



	R1	R2	R3	R4	R5	R6
α-Neu5Ac2Me	COOH	OCH_3	OH	O	CH_3	OH
β-Neu5Ac2Me	OCH_3	COOH	OH	O	CH_3	OH
α-Neu5thioAc2Me	COOH	OCH_3	OH	S	CH_3	OH
α-Neu5thioAc2SMe	COOH	SCH_3	OH	S	CH_3	OH
α-Neu5Prop2Me	COOH	OCH_3	OH	O	C_2H_5	OH
α-Neu5thioProp2Me	COOH	OCH_3	OH	S	C_2H_5	OH
α-Neu5Acryl2Me	COOH	OCH_3	OH	O	C_2H_3	OH
α-Neu5Mecar2Me	COOH	OCH_3	OH	O	$NHCH_3$	OH
α-Neu5Prop2Bn	COOH	$OCH_2C_6H_5$	OH	O	C_2H_5	OH
α-Neu5FAc2Bn	COOH	$OCH_2C_6H_5$	OH	O	CH_2F	OH
α-Neu4,5Ac2Bn	COOH	$OCH_2C_6H_5$	$OCOCH_3$	O	CH_3	OH
α-Neu4,5,7Ac3Bn	COOH	$OCH_2C_6H_5$	$OCOCH_3$	O	CH_3	$OCOCH_3$

B



	R2	R4
α-Neu5Ac2 polymer	O	O
α-Neu5thioAc2 polymer	O	S
α-Neu5thioAc2S polymer	S	S

FIG. 1. Structural formulas of *N*-acetylneuraminic acid (Neu5Ac) analogs. (A) α-Neu5Ac2Me: α-2-*O*-methyl-5-*N*-acetylneuraminic acid, β-Neu5Ac2Me: β-2-*O*-methyl-5-*N*-acetylneuraminic acid, α-Neu5thioAc2Me: α-2-*O*-methyl-5-*N*-thioacetylneuraminic acid, α-Neu5thioAc2SMe: α-2-*S*-methyl-5-thioacetylneuraminic acid, α-Neu5Prop2Me: α-2-*O*-methyl-5-*N*-propionylneuraminic acid, α-Neu5thioProp2Me:α-2-*O*-methyl-5-*N*-thiopropionylneuraminic acid, α-Neu5Acryl2Me:α-2-*O*-methyl-5-*N*-acryloylneuraminic acid, α-Neu5Mecar2Me: α-2-*O*-methyl-5-*N*-methylcarbamoylneuraminic acid, α-Neu5Prop2Bn: α-2-*O*-benzyl-5-*N*-propionylneuraminic acid, α-Neu5FAc2Bn: α-2-*O*-benzyl-5-*N*-fluoroacetylneuraminic acid, α-Neu4,5$Ac_2$2Bn: α-2-*O*-benzyl-4-*O*-acetyl-5-*N*-acetylneuraminic acid, α-Neu4,5,7$Ac_3$2Bn: α-2-*O*-benzyl-4,7-di-*O*-acetyl-5-*N*-acetylneuraminic acid. The small numbers indicate the position of carbon on the main chain of Neu5Ac (B) α-Neu5Ac2 polymer, α-Neu5thioAc2 polymer, and α-Neu5thioAc2S polymer are polymers of α-5-*N*-acetylneuraminic acid or α-5-*N*-thioacetylneuraminic acid conjugated to an acrylamide chain through α-2-*O* or α-2-*S* binding.

oylamidoethylthio)propyl group of the respective *N*-acetylneuraminic acid. Copolymerization with acrylamide was performed under nitrogen, employing ammonium peroxodisulfate as the starter and tetramethylethylenediamine as the activator. Unless mentioned otherwise the initial molar ratio of acrylamide and sialic acid analog amounted to 5:1. Practically the same ratio was found in the polymers. The molecular weight of the polymers was 165,000 ± 30,000. The detailed synthesis of the polymers will be described elsewhere (P. Hetterich and R. Brossmer, manuscript in preparation).

Competitive binding experiments

The affinity of the sialic acid analogs to influenza viruses was evaluated in a competitive binding assay. The assay was based on the inhibition of binding of bovine fetuin to immobilized virus by the sialic acid analogs. Each well of plastic plates (Microelisa plates, Immuron, Dynatech) was coated overnight at 4° with purified X-31 influenza virus in 50 μl of phosphate-buffered saline (PBS) at a protein concentration of 5 mg/ml, washed with PBS containing 0.05% Tween 20 (PBS–Tween), and blocked with PBS–Tween containing 1% bovine serum albumin (PBS–BSA–Tween). Mixtures of various concentrations of sialic acid analogs with 10 n*M* fetuin in PBS–BSA–Tween were incubated in the virus-coated wells for 1 hr at 4°. After washing with PBS–Tween, rabbit anti-fetuin serum was added to each well. After incubation for 1 hr at room temperature, samples were washed with PBS–Tween, and peroxidase-conjugated anti-rabbit IgG goat serum was added. After incubation for 1 hr at room temperature the amount of bound conjugate was quantified by the standard assay of peroxidase activity using *O*-phenylenediamine-2HCl. To correct for nonspecific binding, several wells on each plate were coated with noninfected allantoic fluid, and the mean absorbency was used as the background value. The affinity of sialic acid analogs to influenza virus was measured as the inhibition of binding of fetuin, using the absorbency in the absence of the competing sialic acid analogs and that in the absence of fetuin as 0 and 100% inhibition, respectively.

Inhibition of virus replication

The effects of the sialic acid analogs on virus replication were examined by a plaque test. Madin–Darby canine kidney (MDCK) cells in plastic dishes of 3.5-cm diameter were inoculated with about 100 PFU of influenza virus and incubated at 37° in Eagle's minimum essential medium containing 1% agarose in the presence of various concentrations of sialic acid analogs. The effect of the analogs was measured as the reduction of plaque numbers occurring in the presence of analogs.

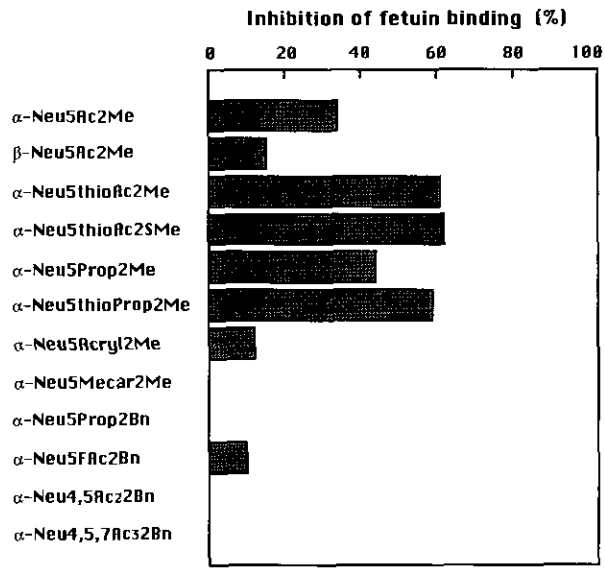


FIG. 2. Inhibitory effect of sialic acid analogs on the binding of fetuin to influenza virus X-31. Binding of fetuin to immobilized X-31 virus in the presence of a 10 m*M* analog was assayed by ELISA as described under Materials and Methods. The affinity of each sialic acid compound to the virus was measured by inhibition of fetuin binding. Representative results of four experiments are shown.

Viral neuraminidase treatment of sialic acid analogs

Sialic acid analog (0.4 mg) or sialic acid analog containing polymer (0.5 mg) was dissolved in distilled water (120 μl). After addition of 30 μl of a suspension of X-31 virus in PBS (50,000 HAU) the mixture was incubated at 37°. The reaction was followed by thin-layer chromatography on silica gel (E. Merck, Darmstadt, Germany), employing the solvent 7:3 1-propanol:water. Degradation was estimated by spraying with 2 *N* sulfuric acid followed by heating at 200°.

RESULTS

Binding affinity of sialic acid analogs to HA on virus particles

The importance of the 5-*N*-acetyl group of Neu5Ac for binding to the RBS of HA was shown by direct binding experiments and by the fact that influenza virus strains differ in recognition of Neu5Ac and Neu5Gc (Higa *et al.*, 1985; Suzuki *et al.*, 1986, 1992; Nobusawa *et al.*, 1991). In search of a substance with increased HA affinity, we synthesized several sialic acid analogs modified at the 5-*N*-acetyl group, of which Neu5thioAc2Me had the highest binding activity for bromelain-treated HA, with a K_d of 0.1 m*M* compared to a K_d of 1.5 m*M* Neu5Ac2Me, when analyzed by ^{1}H NMR spectroscopy (Machytka *et al.*, 1993). It was then of interest to test the affinity of this compound to native HA incorporated into virus particles. A competitive binding assay with fetuin to HA was used for this purpose.

Figure 2 shows the inhibitory effects of the sialic acid

analogs on the binding of fetuin to influenza virus. Neu5Ac2Me and Neu5Prop2Me caused about 35 and 45% inhibition, respectively, whereas the inhibitory effect was less expressed with Neu5Acryl2Me and Neu5Mecar2Me. Introduction of a thioacetamide or a thiopropionylamide group increased the inhibition to about 60% as shown with Neu5thioAc2Me and Neu5thioProp2Me. These results are compatible with those obtained in the ^{1}H NMR studies (Machytka *et al.*, 1993). β-Neu5Ac2Me, which has a different space orientation at *C*2, has a lower inhibitory effect, which is compatible with the results of other laboratories (Pritchett *et al.*, 1987; Sauter *et al.*, 1989; Kelm *et al.*, 1992; Matrosovich *et al.*, 1993). Substitution of the methyl group at *C*2 by a benzyl group and/or the introduction of acetyl groups at *C*4 and *C*7 did not increase the inhibitory effect. On the other hand, comparison of Neu5thioAc2SMe with Neu5thioAc2Me indicates that the relatively high inhibitory effect remained when a thioglycosidic linkage was present at *C*2. Unlike the results reported by Matrosovich *et al.* (1993), a decrease of interfering activity was not observed when *C*2 oxygen was replaced by sulfur.

Polyacrylamide-based polymers of sialic acid are more effective inhibitors of receptor binding than their monovalent analogs (Matrosovich *et al.*, 1990; Spaltenstein and Whitesides, 1991). Neu5Ac, Neu5thioAc, and Neu5thioAc2S were therefore conjugated via a spacer to polyacrylamide chains (Fig. 1B), and the affinity of the polymers to HA was evaluated by a competitive binding assay. As shown in Fig. 3, the polymers inhibited fetuin binding to HA at about 10^3 times lower concentrations than the respective monomers, which is compatible with the results of Matrosovich *et al.* (1990). The Neu5thioAc2 and Neu5thioAc2S polymers inhibited the binding of fetuin at a concentration of 0.0016 m*M*, whereas the Neu5Ac2 polymer had no effect at the same concentration. This shows that the enhanced intrinsic binding affinity of thioacetamide was preserved after polymerization. However, even at the high concentration of 5 m*M*, the polymers did not block the binding of fetuin completely.

Degradation of sialic acid analogs by viral NA

The binding experiments shown in Figs. 2 and 3 were performed at 4° to prevent degradation of the inhibitors by viral NA. It is reasonable to assume that sialic acid analogs resistant to NA should be particularly suitable for application as inhibitors of virus replication at around 37°. It was therefore of interest to find out if Neu5thioAc2SMe, which exhibited the same level of affinity to HA as Neu5thioAc2Me, is NA resistant. Table 1 shows that this was indeed the case. After 4 hr incubation at 37° with X-31, the monomeric and the polymeric *O*-glycoside were cleaved up to 90%. On the other hand, the *S*-glycoside of the Neu5thioAc2SMe and the Neu5thioAc2S polymers was not destroyed even after 24 hr.

Inhibition of virus replication

As a next step, the inhibitory effect of the sialic acid polymers on virus replication was studied by plaque-reduction assays (Fig. 4). Virus replication was inhibited with considerably higher efficiency than fetuin binding. Neu5Ac2 polymer showed complete inhibition of plaque formation of X-31 at a concentration of 2 μM Neu5Ac. The Neu5thioAc2 polymer completely suppressed plaque formation at a 10-fold lower concentration, 0.2 μM Neu5thioAc, reflecting the strong interference of this inhibitor with binding of HA to cellular receptors. The Neu5thioAc2S polymer showed as strong an inhibition as did the Neu5thioAc2 polymer on X-31 (*Table 2*).

The effects of the Neu5Ac2 polymer, the Neu5thioAc2 polymer, and the Neu5thioAc2S polymer on the replication of other influenza virus strains were also examined. Table 2 shows the concentration of each polymerized sialic acid analog at which plaque formation is reduced by 50%. Some remarkably distinctive effects of the three polymers on the growth of each of the strains were observed. The Neu5Ac2 polymer did not interfere with the infection of any of the other four strains. On the other hand, the Neu5thioAc2 polymer reduced the plaque number of WSN and FPV by 50% at 100 and 200 μM, respectively. The Neu5thioAc2S polymer showed the widest interference, since it had effects on the replication of all strains, except for strain Singapore. These observations demonstrate that introduction of sulfur into the *N*-acetyl group increased the sensitivity of three of the five strains analyzed and that the NA resistance of the Neu5thioAc2S polymer enhanced its inhibitory effect on virus replication also with three strains. None of the polymers inhibited plaque formation of vesicular stomatitis virus, showing that they have no affect on the host cells at the concentrations used in these studies.

We have also analyzed if the sialic acid content of the polymers has an effect on their inhibitory potential. To this end, Neu5thioAc2 polymer preparations with ratios of Neu5thioAc to acrylamide of 1:2 and 1:10 were compared with the standard polymer, containing the ratio 1:5, in plaque-reduction assays. No differences were observed (data not shown).

DISCUSSION

The insight into the molecular details of receptor recognition of influenza viruses provides a rational basis for designing receptor analogs as inhibitors of virus infection. The data presented here confirm the importance of the 5-*N*-acetyl group of Neu5Ac in the interaction with the RBS that has already been established in other studies (Higa *et al.*, 1985; Weis *et al.*, 1988; Kelm *et al.*, 1992; Sauter *et al.*, 1992; Matrosovich *et al.*, 1993). The atomic model of the HA–Neu5Ac interaction that has emerged from X-ray crystallographic studies shows Van der Waals

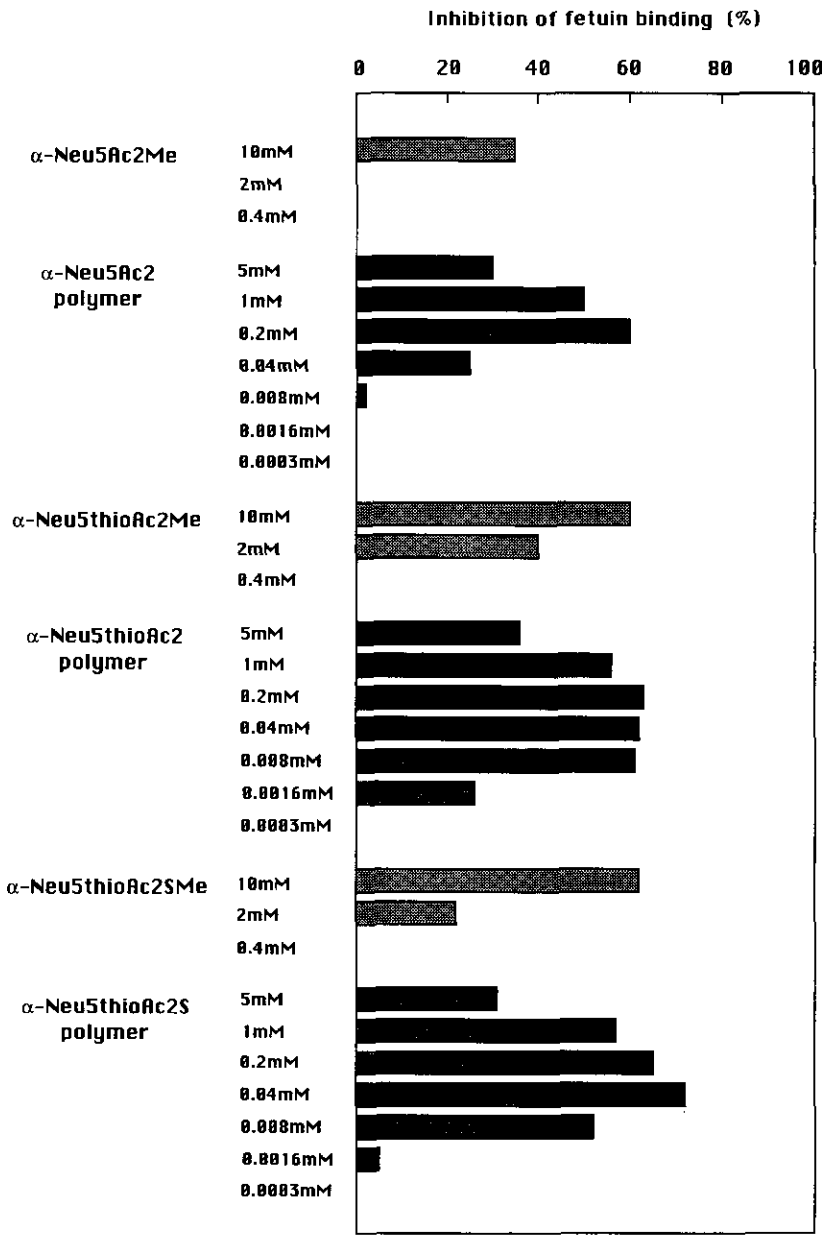


FIG. 3. Increased inhibition of the binding of fetuin to HA by polymerized sialic acid analogs. Fetuin (10 n*M*) was mixed with α-Neu5Ac2Me, α-Neu5thioAc2Me, and α-Neu5thioAc2SMe or with polymers of each monomer, α-Neu5Ac2 polymer, α-Neu5thioAc2 polymer, and α-Neu5thioAc2S polymer, at the various concentrations indicated and incubated with immobilized X-31. The amount of fetuin bound to virus was assayed as described under Materials and Methods. The concentration corresponds to that of the sialic acid analogs linked to the acrylamide main chain. Representative results of five experiments are shown.

contact between the 5-*N*-acetyl group and the indole ring of Trp-153 as well as Gly-134 (Weis *et al.*, 1988). These two amino acids are conserved in all known type A and B influenza viruses (Weis *et al.*, 1988; Nobusawa *et al.*, 1991; Sauter *et al.*, 1992). Various kinds of analogs with modifications in this position have thus far been examined for their affinity to HA; however, in most cases, lower affinity was observed (Higa *et al.*, 1985; Pritchett *et al.*, 1987; Kelm *et al.*, 1992; Sauter *et al.*, 1992). Sauter *et al.* (1992) reported that only Neu5Prop2Me possesses affinity similar to that of Neu5Ac2Me through the interaction of its 5-*N*-propionyl group with the indole ring of Trp-153. In the same report they demonstrated that, although $C^{\delta 2}$ of Leu-194 is the closest protein atom to both the carbonyl oxygen of the *N*-acetyl group (3.3 Å away) and the hydroxyl oxygen of *C*7 (3.9 Å away), neither ligand atom makes hydrogen bonds to HA. They proposed the replacement of oxygen by sulfur, which would preserve the sp^2 character of the carbonyl carbon, yet decrease its polarity to maximize hydrophobic interactions with Leu-194 for high affinity to HA. In our previous report we demonstrated that substitution of the carbonyl oxygen in

TABLE 1

Cleavage of Sialic Acid Analogs by Virus NA

Sialic acid analogs	Cleaved compound (%)
α-Neu5Ac2Me	~90
α-Neu5thioAc2Me	~90
α-Neu5thioAc2SMe	0
α-Neu5Ac2 polymer	~90
α-Neu5thioAc2 polymer	~90
α-Neu5thioAc2S polymer	0

the *N*-acetyl group by sulfur resulted in increased affinity of Neu5thioAc2Me to HA solubilized by bromelain treatment (Machytka *et al.*, 1993). As shown in Fig. 2, Neu5thioAc2Me, as well as Neu5thioProp2Me, bound to natural HA on virus particles again with higher affinity than each sialic acid counterpart without sulfur. Our results verified the prediction of Sauter *et al.* (1992), but direct proof of the interaction between the thioamide and Leu-194 requires X-ray analysis.

Polymerization had no affect on the essentially increased binding affinity of Neu5thioAc2Me and Neu5thioAc2SMe, since the polymers of these analogs exhibited again 5–10 times higher affinities than did the Neu5Ac polymer (Fig. 3). The observation indicates that the increased binding potency caused by polymerization resulted from a different mechanism, namely, the cooperative effects of multiple interactions between the sialic acid residues on the polymer and the HA molecules on an influenza virus particle (Morawiecki and Lisowska, 1965; Whitehead and Winzler, 1968; Barclay *et al.*, 1969; Hanaoka *et al.*, 1989; Pritchett and Paulson, 1989; Matrosovich *et al.*, 1990; Glick and Knowles, 1991; Sabesan *et al.*, 1991; Spaltenstein and Whitesides, 1991). Glick *et al.* (1991) showed that bivalent sialic acid with a spaced length of 55 Å sharply increased the binding affinity to virions. From these results, they calculated the distance between adjacent RBSs on different HA trimers to be less than 55 Å. Optimal binding capacity of α2-macroglobulin also depends on the distribution of the sialic acid containing oligosaccharides on its surface (Pritchett and Paulson, 1989). However, in the present study we were, within the limits of our experimental design, not able to see a correlation between inhibiting capacity and sialic acid content of the polymers.

It is noteworthy that polymers inhibited the binding of fetuin only up to 65% even at high concentrations (Fig. 3), whereas they completely suppressed the replication of the virus at about 100 times lower concentrations (2 and 0.2 μ*M* for the Neu5Ac2 polymer and the Neu5thioAc2 polymer, respectively) (Fig. 4). This phenomenon probably reflects self-competition of the polymer as well as differences in molecular size or molecular shape between fetuin and sialic acid containing virus receptors on the cell. Because of steric hinderance, the polymer may not be able to bind to all RBSs on the influenza virus particle even if present in a high concentration. Again for steric reasons, the unoccupied RBSs may be accessible for fetuin, but not for sialic acid containing glycoproteins or gangliosides on the cell. It is therefore conceivable that, without binding to all of the RBSs on the virion, the polymer completely blocks virus attachment to cell receptors.

Recently, Matrosovich *et al.* (1993) showed that the X-31 strain has a specificity of sialic acid recognition that is different from that of other strains. When we analyzed in the present study the inhibition of virus replication by the Neu5thioAc2 polymer, the effect was more than 5000 times higher with X-31 than with other strains. This may reflect the peculiar specificity of X-31 in receptor recognition. However, the observation that the Neu5thioAc2 polymer also has an inhibitory effect on other influenza virus strains stresses the importance of the sulfur atom in the thioacetamide group for binding to HA.

So far little has been known about the effect of NA degradation on the ability of sialic acid analogs to interfere with virus replication. It was therefore of interest to analyze the inhibitory capacity of a compound showing NA resistence, which was accomplished by the introduction of a thioglycosidic linkage. When compared to the Neu5thioAc2 polymer, the Neu5thioAc2S polymer had a 10,000 times higher inhibitory effect on replication of strain Pennsylvania. In contrast, there was no difference between both compounds when used with strain Singapore. Strain Pennsylvania differed from strain Singapore also by a 40 times higher specific NA activity (data not shown). These observations clearly prove that resistance against NA is an intrinsic advantage for the inhibitor efficiency, especially in the case of influenza virus strains

TABLE 2

Concentration of Sialic Acid Analogs Conjugated to Acrylamide Polymers That Exhibits 50% Reduction of Plaque Formation of Various Influenza Virus Strains[a]

Virus	Concentration (μ*M*)[b]		
	α-Neu5Ac2 polymer	α-Neu5thioAc2 polymer	α-Neu5thioAc2S polymer
X-31 (H3N2)	0.2	0.02	0.02
A/WSN/33 (H1N1)	>400	100	5
A/Singapore/ 1/57 (H2N2)	>400	>400	>400
A/chick/Penn/83 (H5N2)	>400	>400	0.05
A/FPV/Rostock/ 34 (H7N1)	>400	200	100

[a] Representative results of four experiments are shown.

[b] The concentration corresponds to that of sialic acid analogs linked to the polyacrylamide backbone.

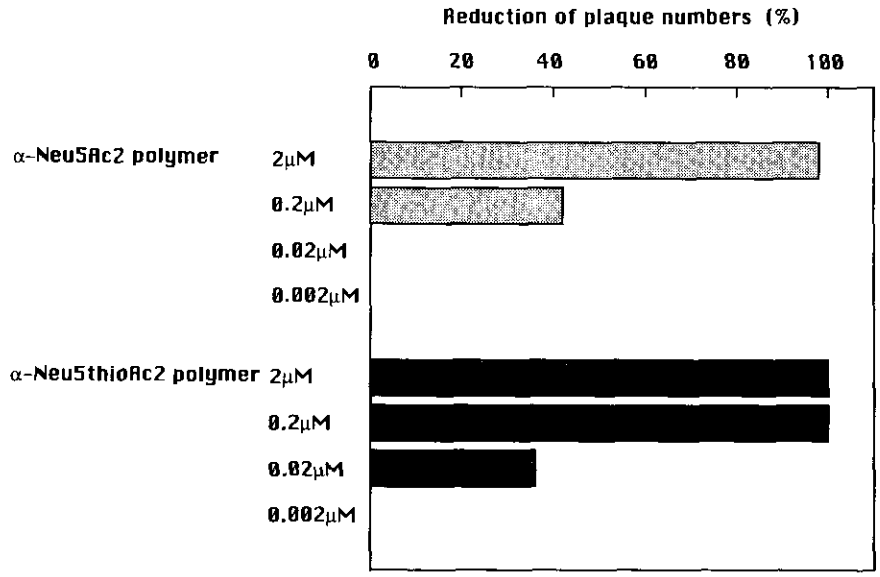


FIG. 4. Suppression of virus replication by polymers of sialic acid analogs conjugated to acrylamide. MDCK cells were inoculated with about 100 PFU/well of X-31 and incubated with MEM medium containing 1% agarose in the presence of the sialic acid polymer at the final concentrations indicated. The inhibitory effect of the compounds on virus replication is indicated as the reduction of the plaque number. The concentration corresponds to that of the sialic acid analogs linked to the acrylamide main chains. Representative results of four experiments are shown.

with strong NA activity. The results also indicate that the Neu5thioAc2S polymer has the potential to be used as an inhibitor of many influenza virus strains.

ACKNOWLEDGMENTS

M.I. was a recipient of a fellowship of the Alexander von Humboldt Foundation. This work was supported by the Deutsche Forschungsgemeinschaft (SFB 286) and by the Fonds der Chemischen Industrie.

REFERENCES

Barclay, G. R., Flewett, T. H., Keller, E., Halsall, H. B., and Spragg, S. P. (1969). Effect of polymerized orosomucoid on some strains of influenza virus. *Biochem. J.* **111,** 353–357.

Bergelson, L. D., Bukrinskaya, A. G., Prokazova, N. V., Shaposhnikova, G. I., Kocharov, S. L., Shevchenko, V. P., Kornilaeva, G. V., and Fomina-Ageeva, E. V. (1982). Role of gangliosides in reception of influenza virus. *Eur. J. Biochem.* **128,** 467–474.

Glick, G. D., and Knowles, J. R. (1991). Molecular recognition of bivalent sialosides by influenza virus. *J. Am. Chem. Soc.* **113,** 4701–4703.

Glick, G. D., Toogood, P. L., Wiley, D. C., Skehel, J. J., and Knowles, J. R. (1991). Ligand recognition by influenza virus. *J. Biol. Chem.* **266,** 23660–23669.

Hanaoka, K., Pritchett, T. J., Takasaki, S., Kochibe, N., Sabesan, S., Paulson, J. C., and Kobata, A. (1989). 4-*O*-Acetyl-*N*-acetylneuraminic acid in the *N*-linked carbohydrate structures of equine and gunia pig α2-macroglobulins, potent inhibitors of influenza virus infection. *J. Biol. Chem.* **264,** 9842–9849.

Higa, H. H., Rogers, G. N., and Paulson, J. C. (1985). Influenza virus hemagglutinins differentiate between receptor determinants bearing *N*-acetyl-, *N*-glycollyl-, and *N,O*-diacetylneuraminic acids. *Virology* **144,** 279–282.

Isecke, R., and Brossmer, R. (1994). Synthesis of 5-*N*- and 9-*N*-thioacylated sialic acids. *Tetrahedron* **50,** 7445–7460.

Kelm, S., Paulson, J. C., Rose, U., Brossmer, R., Schmid, W., Bandgar, B. P., Schreiner, E., Hartmann, M., and Zbiral, E. (1992). Use of sialic acid analogues to define functional groups involved in binding to the influenza virus hemagglutinin. *Eur. J. Biochem.* **205,** 147–153.

Kuhn, R., Lutz, P., and MacDonald, D. L. (1966). Synthese anomerer Sialinsäure-methylketoside. *Chem. Ber.* **99,** 611–617.

Laver, W. G., and Kilbourne, E. D. (1966). Identification in a recombinant influenza virus of structural proteins derived from both parents. *Virology* **30,** 493–501.

Machytka, D., Kharitonenkov, I., Isecke, R., Hetterich, P., Brossmer, R., Klein, R. A., Klenk, H.-D., and Egge, H. (1993). Methyl α-glycoside of *N*-thioacetyl-D-neuraminic acid: A potential inhibitor of influenza A virus. *FEBS Lett.* **334,** 117–120.

Matrosovich, M. N., Mochalova, L. V., Marinina, V. P., Byramova, N. E., and Bovin, N. V. (1990). Synthetic polymeric sialoside inhibitors of influenza virus receptor-binding activity. *FEBS Lett.* **272,** 209–212.

Matrosovich, M. N., Gambaryan, A. S., Tuzikov, A. B., Byramova, N. E., Mochalova, L. V., Golbraikh, A. A., Schinderovich, M. D., Finne, J., and Bovin, N. V. (1993). Probing of the receptor-binding sites of the H1 and H3 influenza A and B virus hemagglutinins by synthetic and natural sialosides. *Virology* **196,** 111–121.

Morawiecki, A., and Lisowska, E. (1965). Polymerized orosomucoid; an inhibitor of influenza virus hemagglutinin. *Biochem. Biophys. Res. Commun.* **18,** 606–610.

Nobusawa, E., Aoyama, T., Kato, H., Suzuki, Y., Tateno, Y., and Nakajima, K. (1991). Comparison of complete amino acid sequences and receptor-binding properties among 13 serotypes of hemagglutinins of influenza A viruses. *Virology* **182,** 475–485.

Paulson, J. C., Sadler, J. E., and Hill, R. L. (1979). Restoration of specific myxovirus receptors to asialoerythrocytes by incorporation of sialic acid with pure sialytransferases. *J. Biol. Chem.* **254,** 2120–2124.

Paulson, J. C. (1985). Interactions of animal viruses with cell surface receptors. *In* "The Receptors" (M. Conn, Ed.), Vol. 2, pp. 131–219. Academic Press, Orlando, FL.

Pritchett, T. J., Brossmer, R., Rose, U., and Paulson, J. C. (1987). Recognition of monovalent sialosides by influenza virus H3 hemagglutinin. *Virology* **160,** 502–506.

Pritchett, T. J., and Paulson, J. C. (1989). Basis for the potent inhibition of influenza virus infection by equine and guinea pig α2-macroglobulin. *J. Biol. Chem.* **264,** 9850–9858.

Rogers, G. N., and Paulson, J. C. (1983). Receptor determinant of human and animal influenza virus isolates: Differences in receptor specificity of the H3 hemagglutinin based on species of origin. *Virology* **127**, 361–373.

Ruigrok, R. W., Andree, P. J., Hoof Van Huysduynen, R. A. M., and Mellema, J. E. (1984). Characterization of three highly purified influenza virus strains by electron microscopy. *J. Gen. Virol.* **65**, 799–802.

Sabesan, S., Bock, K., and Paulson, J. C. (1991). Conformational analysis of sialyloligosaccharides. *Carbohydr. Res.* **218**, 27–54.

Sauter, N. K., Bednarsky, N. D., Wursburg, B. A., Hanson, J. E., Whitesides, G. M., Skehel, J. J., and Wiley, D. C. (1989). Hemagglutinins from two influenza virus variants bind to sialic acid derivatives with millimolar dissociation constants: A 500-MHz proton nuclear magnetic resonance study. *Biochemistry* **28**, 8388–8396.

Sauter, N. K., Hanson, J. E., Glick, G. D., Brown, J. H., Crowther, R. L., Seong-Joon, P., Skehel, J. J., and Wiley, D. C. (1992). Binding of influenza virus hemagglutinin to analogs of its cell-surface receptor, sialic acid: Analysis by proton nuclear magnetic resonance spectroscopy and X-ray crystallography. *Biochemistry* **31**, 9609–9621.

Spaltenstein, A., and Whitesides, G. M. (1991). Polyacrylamides bearing pendant α-sialoside groups strongly inhibit agglutination of erythrocytes by influenza virus. *J. Am. Chem. Soc.* **113**, 686–687.

Suzuki, Y., Matsusnaga, M., and Matsumoto, M. (1985). *N*-Acetylneuraminyl-lactosylceramide, GM3-NeuAc, a new influenza A virus receptor which mediates the adsorption-fusion process of viral infection: Binding specificity of influenza virus A/Aichi/2/68 (H3N2) to membrane-associated GM3 with different molecular species of sialic acid. *J. Biol. Chem.* **260**, 1362–1365.

Suzuki, Y., Nagao, Y., Kato, H., Matsumoto, M., Nerome, K., Nakajima, K., and Nobusawa, E. (1986). Human influenza A virus hemagglutinin distinguishes sialyloligosaccharides in membrane-associated gangliosides as its receptor which mediates the adsorption and fusion processes of virus infection: Specificity for oligosaccharides and sialic acids and the sequence to which sialic acid is attached. *J. Biol. Chem.* **261**, 17057–17061.

Suzuki, Y., Nakao, T., Ito, T., Watanabe, N., Toda, Y., Guiyun, X., Suzuki, T., Kobayashi, T., Kimura, Y., Yamada, A., Sugawara, K., Nishimura, H., Kitame, F., Nakamura, K., Deya, E., Kiso, M., and Hasegawa, A. (1992). Structural determination of gangliosides that bind to influenza A, B, and C viruses by an improved binding assay: Strain-specific receptor epitopes in sialo-sugar chains. *Virology* **189**, 121–131.

Tiffany, J. M., and Blough, H. A. (1970). Estimation of the number of surface projections on myxo- and paramyxoviruses. *Virology* **41**, 392–394.

Weis, W., Brown, J. H., Cusack, S., Paulson, J. C., Skehel, J. J., and Wiley, D. C. (1988). Structure of the influenza virus hemagglutinin complexed with its receptor, sialic acid. *Nature* **333**, 426–431.

Whitehead, P. H., and Winzler, R. J. (1968). Inhibition of viral hemagglutinin by aggregated orosomucoid. *Arch. Biochem. Biophys.* **126**, 657–663.